Norepinephrine Causes a Biphasic Change in Mammalian Pinealocye Membrane Potential: Role of α_{1B}-Adrenoreceptors, Phospholipase C, and Ca^{2+}

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Perforated patch clamp recording was used to study the control of membrane potential (V_m) and spontaneous electrical activity in the rat pinealocyte by norepinephrine. Norepinephrine did not alter spiking frequency. However, it was found to act through α_{1B}-adrenoreceptors in a concentration-dependent manner (0.1–10 μM) to produce a biphasic change in V_m. The initial response was a hyperpolarization (~13 mV from a resting potential of −46 mV) due to a transient (~5 sec) outward K^+ current (~50 pA). This current appears to be triggered by Ca^{2+} released from intracellular stores, based on the observation that it was also seen in cells bathed in Ca^{2+}-deficient medium. In addition, pharmacological studies indicate that this current was dependent on phospholipase C (PLC) activation and was in part mediated by bicuculline methiodide and apamin-sensitive Ca^{2+}-controlled K^+ channels. The initial transient hyperpolarization was followed by a sustained depolarization (~4 mV) due to an inward current (~10 pA). This response was dependent on PLC-dependent activation of Na^+/Ca^{2+} influx but did not involve nifedipine-sensitive voltage-gated Ca^{2+} channels. Together, these results indicate for the first time that activation of α_{1B}-adrenoreceptors initiates a PLC-dependent biphasic change in pinealocyte V_m characterized by an initial transient hyperpolarization mediated by a mixture of Ca^{2+}-activated K^+ channels followed by a sustained depolarization mediated by a Ca^{2+}-conducting nonselective cation channel. These observations indicate that both continuous elevation of intracellular Ca^{2+} and sustained depolarization at approximately −40 mV are associated with and are likely to be required for activation of the pinealocyte. **(*Endocrinology* 152: 3842–3851, 2011)**

The pineal gland is a neuroendocrine transducer, which generates a nocturnal increase in melatonin production, reflecting stimulation by norepinephrine (NE). This temporal pattern is controlled by the master circadian oscillator located in the suprachiasmatic nucleus; light acts on this system to block stimulation of the pineal gland and to reset the clock (1–3). NE is the primary physiological transmitter of the pineal gland (3–5) and acts via two adrenergic receptors that are highly expressed in this tissue: the G_q-coupled α_{1B}-adrenergic receptors (6, 7) and the G_s-coupled β_1-adrenergic receptors (8). The coincident activation of both receptors controls a large (~100-fold) increase in cAMP. β_1-Adrenergic activation causes an increase in adenylate cyclase activity through $G_s\alpha$. This is potentiated by α_{1B}-adrenoreceptor activation through a mechanism dependent on an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and phospholipase C (PLC) activity, leading to activation of protein kinase C (6,

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Abbreviations: AP, Action potential; BK, big potassium; BMI, bicuculline methiodide; $[Ca^{2+}]_i$, intracellular calcium concentration; Ca_v, voltage-gated Ca^{2+}; HP, holding potential; IBTX, iberiotoxin; IK, intermediate conductance; IP_3, inositol trisphosphate; ISOP, isoproterenol; K_{Ca}, Ca^{2+}-controlled K^+; Na_v, voltage-gated Na^+; IK, intermediate potassium; Kv7/KCNQ, potassium voltage-gated and cyclic nucleotide-regulated channel, subfamily Q, member 7; NE, norepinephrine; $NMDG^+$, N-methyl-D-glucamine; PE, phenylepinephrine; PLC, phospholipase C; SK, small potassium; TRAM 34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U73343, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione; V_m, membrane potential; XE991, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride.

8–12). This results in activation of protein kinase A and phosphorylation of cAMP-response element-binding protein (3, 13).

As a consequence of this, expression of *Aanat* is induced. This gene encodes the enzyme responsible for large changes in melatonin production, arylalkylamine N-acetyltransferase (4). Induction of *Aanat* is further enhanced by the α_{1B}-adrenoreceptor-dependent increase in $[Ca^{2+}]_i$ (14). Protein kinase A activation not only induces *Aanat* expression but also results in AANAT phosphorylation. This triggers binding of phosphorylated AANAT to 14-3-3 protein, which stabilizes the enzyme and increases serotonin affinity (4), resulting in an increase in melatonin production. Melatonin is highly lipophilic, and because of this, the increase in melatonin production leads to an immediate increase in melatonin secretion.

The pineal gland is composed 95% of the melatonin synthesizing pinealocytes. These cells express numerous plasma membrane channels as revealed by electrophysiological studies, including voltage-gated Ca^{2+} (Ca_v) and voltage-gated Na^+ (Na_v) channels, two types of Ca^{2+}-controlled K^+ (K_{Ca}) channels, and at least two types of voltage-activated K^+ channels (7, 15–21). Rat pinealocytes fire action potentials (AP) spontaneously, with the firing rate being low during the day time and high during the night time (22–25). The classic stimulus-secretion coupling, which controls release of compounds stored in secretory vesicles, is not known to significantly influence melatonin secretion, which, as indicated above, is regulated by production.

In some neuroendocrine cells (*e.g.* TSH-releasing hormone-stimulated pituitary lactotrophs), Ca^{2+} mobilization initiates a transient hyperpolarization due to activation of K_{Ca} channels, followed by a sustained depolarization that permits firing of AP and facilitation of Ca_v influx (26). Application of NE also induces hyperpolarization of the pinealocyte (27–29) and stimulates K^+ efflux, most probably through big potassium (BK) K_{Ca} channels, as identified in pinealocytes using single channel recording (21). Here, we have focused on the NE regulation of membrane potential (V_m), with the goal of determining the sustained effects of NE on V_m because of the importance that electrochemical gradients play in cell physiology. We also were interested in establishing the nature of currents mediating changes in V_m and the signaling pathways controlling these currents. To address these issues, we have studied isolated rat pinealocytes using amphotericin-perforated patch clamp recording (16, 30). This method is superior to those previously used to study pineal V_m, because it establishes better electrical continuity between the pipette buffer and cytoplasm without the loss of cytoplasmic molecules necessary for the maintenance of NE responsivity. The results of these studies are presented below.

Materials and Methods

Chemicals

Iberiotoxin (IBTX), tetrodotoxin citrate, apamin, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM 34), noradrenaline (NE), phenylepinephrine (PE), epinephrine, isoproterenol (ISOP), prazosin, 10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone dihydrochloride (XE991), nifedipine, and bicuculline methiodide (BMI) were purchased from Tocris-Cookson (Bristol, UK); 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione (U73343), and all other chemicals were from Sigma (St. Louis, MO).

Animals and preparation of cultured pinealocytes

Postpubertal Sprague Dawley rats obtained from Taconic Farms (Germantown, NY), which were kept for 7–14 d under a controlled 12-h light, 12-h dark cycle. All experiments were approved by the National Institute of Child Health and Human Development Animals Care Committee. Pinealocytes from male and female rats (aged 7–9 wk) were prepared as previously described (15) with minor modifications. Pineal glands removed between 1000 and 1100 h, dissected free of adhering tissue, washed in ice-cold DMEM without serum (DMEM with high glucose; GIBCO-BRL, Grand Island, NY), incubated in papain (5 min at 37 C, Papain Dissociation System kit; Worthington Biochemical, Lakewood, NJ), and dissociated using gentle titration. Incubation in papain and titration were repeated five to six times to dissociate all cells. Papain inhibitor was added, and the cell preparation was transferred into DMEM containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (all from Life Technologies, Inc., Invitrogen, Carlsbad, CA), glutamine (1% Gluta MAX), L-ascorbic acid (0.1 μg/ml), 9-Cis retinoic acid (10^{-8} M), and triiodothyronie (10^{-8} M) (Sigma). Cells were filtered through nylon mesh (40-μm pores Cell Strainer; BD Falcon, San Jose, CA) and centrifuged ($\sim$1000 $\times$ *g* for 5 min, room temperature). The resulting pellet was resuspended in DMEM containing 10% fetal calf serum. Approximately 150,000 cells were placed on coverslips coated with 1% poly-L-lysine solution (Sigma) in 35-mm culture dishes (BD Falcon) and maintained at 37 C in a 5% CO_2-containing atmosphere until use (1–3 d).

Electrophysiological recordings

V_m and whole-cell currents were measured using the amphotericin-perforated patch-clamp technique at room temperature, as described previously (16, 30). Briefly, cells were continuously perfused with an extracellular solution containing: 150 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (pH was adjusted to 7.3 with NaOH). In some experiments, bath NaCl was replaced (1:1) by N-methyl-D-glucamine ($NMDG^+$). In others, $CaCl_2$ was omitted and 5 mM EGTA added to bath medium. Patch pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and polished by heat to a tip resistance of 5–7 MΩ. The pipette

solution contained: 70 mM K-aspartate, 70 mM KCl, 3 mM $MgCl_2$, and 10 mM HEPES (pH was adjusted to 7.2 with KOH). Before measurement, amphotericin B was added to the pipette solution from a stock solution (20 mg/ml in dimethyl sulfoxide, always freshly prepared) to obtain a final concentration of 200 μg/ml. Recordings were performed 10 min after seal formation. The holding potential (HP) for NE-induced current recording was at −40 mV, if not otherwise stated. Current-clamp and voltage-clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Data were captured and stored using the pClamp 10 software packages in conjunction with the Digidata 1322A A/D converter (Axon Instruments). The access resistance was monitored throughout each experiment. Only recordings with access resistances between 22 and 45 MΩ (average 28.3 ± 2.2 MΩ) were considered acceptable for analysis of NE-induced currents. Series resistance compensation was not used, and the corrections of V_m for the Donnan potential were ignored. All data have been corrected for liquid junction potential (9.7 mV, positive). Solutions were delivered to the recording chamber by a gravity-driven microperfusion system (ALA Scientific Instruments, Westbury, NY). The application tip was routinely positioned approximately 500 μm to the side and 50 μm above the recorded cell. Less than 200 msec was required for the exchange of solutions around the patched cells. To test effects of drugs, NE was usually applied three times to the same cell; time between each NE application was 3 min.

Data analysis

The NE-induced current amplitudes were measured using the Clampfit 10 software (Axon Instruments). Hyperpolarization was measured as the difference between V_m values immediately before initiation of agonist application and at the peak hyperpolarization response. Depolarization was measured as the difference between V_m values immediately before initiation of agonist application and at the peak depolarization response. All numerical values in the text are reported as mean ± SEM. Significant differences between means were determined by a Student's *t* test; *P* values of at least <0.05 were considered significant.

Results

Resting V_m and electrical excitability

All electrophysiological experiments were done on isolated pinealocytes cultured for 1–3 d. The resting V_m in these cells, as measured using amphotericin-perforated patch, was −46 ± 1 mV (n = 48), in agreement with published reports (28). Spontaneous electrical activity (2, 24, 25, 27) was observed in about 20% of these cells, usually after 2 or 3 d in culture (n = 9) (Fig. 1). Two patterns of spontaneous electrical activity were apparent (Fig. 1A, *inset*): single spiking lasting for 5.6 ± 1.4 msec and plateau bursting with longer duration (570 ± 210 msec). The amplitudes of these spikes were comparable (18 ± 3 mV), and both types of spiking were followed by after hyperpolarization (−5.6 ± 1.4 mV).

When extracellular Na^+ was substituted with $NMDG^+$, a large organic cation, the resting V_m of pineal cells rapidly hyperpolarized (Fig. 1, B and C), reaching the value of −65 ± 2.3 mV (n = 15). The return of bath Na^+ not only resulted in a recovery from hyperpolarization but also a transient depolarization, frequently accompanied by initiation of electrical activity. We observed cell-to-cell variation in the duration of the transient depolarization and that the length of the period of subsequent spontaneous firing of AP changed in parallel (Fig. 1, B *vs.* C).

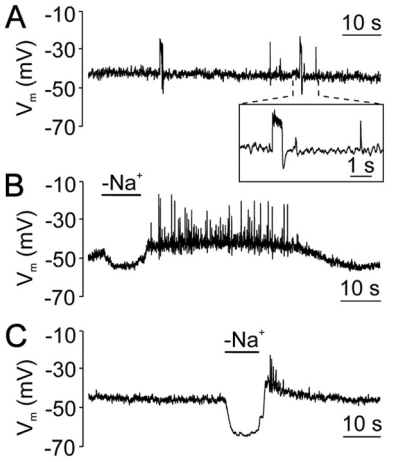

FIG. 1. Electrophysiological properties of single pinealocytes. A, An example of the current-clamp trace with spontaneous electrical activity. The resting V_m was −45 mV. Note single spiking and plateau bursting AP presented on an *expanded time scale*. B and C, Examples of effects of replacing bath Na^+ with $NMDG^+$ on hyperpolarization of the cell membrane. The return of Na^+ to the extracellular bath caused rapid recovery of the resting V_m and transient depolarization accompanied by initiation of electrical activity. In this and following figures, all traces were derived by the amphotericin-perforated patch clamp recording, and no current was injected. *Horizontal bars* indicate duration of Na^+-free conditions.

In contrast, tetrodotoxin, a blocker of Na_v channels, did not affect the resting V_m (data not shown). These results indicate that a large background Na^+ conductance through channel(s) other than Na_v contribute(s) to the resting V_m. Pinealocyte V_m is lower than in other neuroendocrine cells (50–70 mV) (26), thereby driving these pineal cells above threshold for sustained activation of a fraction of Ca_v channels.

Agonist-induced changes in V_m and current

The presence of functional adrenergic receptors was tested by a short (5–10 sec) application of NE in 101 cells. The majority of cells were electrically silent during NE application, AP were rarely observed. Current clamp recordings revealed that 1 μM NE application induced biphasic changes in resting V_m composed of a relatively rapid and transient hyperpolarization (−12.0 ± 2.4 mV) followed by a small depolarization (4.4 ± 0.5 mV) (Fig. 2A), which lasted as long as the agonist was present (Fig. 2C). This was observed with a delay of 2.3 ± 0.2 sec after application in 12 of 18 cells. The depolarization phase or

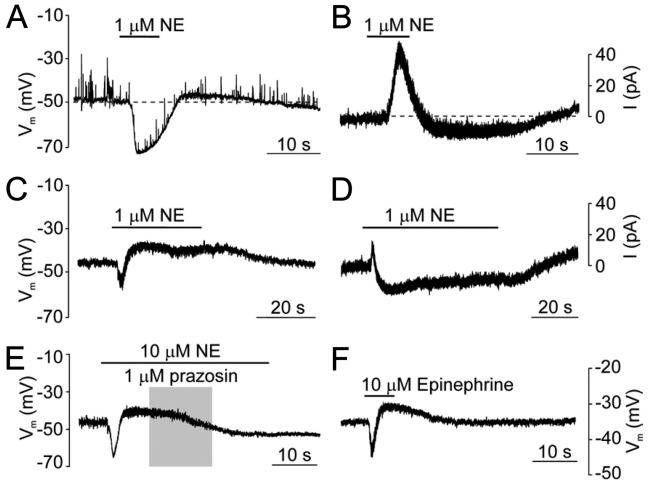

FIG. 2. Characterization of NE-induced responses of single pinealocytes using current clamp and voltage clamp recording. A, Current clamp whole-cell recording of V_m from pinealocytes before, during, and after the application of 1 μM NE. B, Voltage clamp whole-cell recording of NE-evoked current (I) from the same pinealocyte shown in A after a 3-min washout period. Membrane currents were recorded from cells voltage-clamped at −40 mV. The *dotted lines* indicate the resting V_m level (A) and zero current level (B). Note the biphasic nature of voltage and current responses in A and B. C and D, Dependence of the response on duration of NE application. Both the depolarization (C) and inward current (D) persisted during entire NE application period (30 and 60 sec, respectively). E, Inhibition of sustained depolarization by prazosin, an α_{1B}-adrenoceptor antagonist, in the presence of NE. *Shaded area* indicates duration of prazosin (1 μM) application. F, Epinephrine-induced biphasic response.

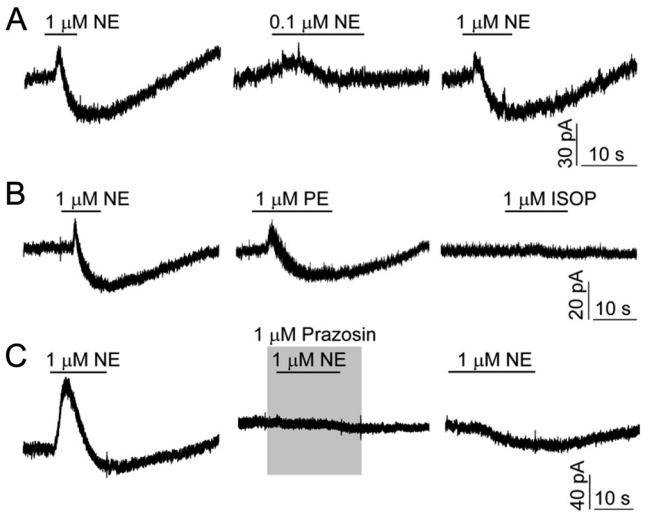

FIG. 3. Role of α_{1B}-adrenoceptors in NE-induced current responses. A, Effects of NE (0.1 and 1 μM) on biphasic current. B, NE (1 μM)- and PE (1 μM)-induced current responses and the lack of response to ISOP (1 μM) to stimulate any current response. C, Inhibition of NE-induced currents by prazosin (1 μM), an α_{1B}-adrenoreceptor antagonist. In all *panels*, the same cells were stimulated three times. The washout period between agonist application was 3 min.

both phases were not detected in six of the 18 cells recorded.

In 56 out of 83 cells (67%) clamped at HP of −40 mV, an NE-induced current was also observed to be comprised of two phases (Fig. 2B): an initial outward current (45 ± 8 pA; peak duration, 6.0 ± 1.1 sec) accompanied by a sustained inward current (−12 ± 1.5 pA; duration, 19 ± 4.8 sec). The inward current was also observed to persist in experiments with longer applications (60 sec) (Fig. 2D). After repetitive stimulation, the amplitude of the NE-induced response diminished and recovery was observed after a 2- to 5-min washing interval (data not shown). Inward or both outward and inward currents were not observed in 27 of the 83 cells studied.

These results reveal for the first time that the NE-induced hyperpolarization of the pinealocyte membrane (27–29) is transient and that this is followed by a sustained depolarization.

α_{1B}-Adrenergic receptors mediate NE actions on outward current

Concentration-dependence studies indicated that the threshold concentration for the NE-evoked outward current was 0.1 μM (Fig. 3A) and the EC_{50} value was approximately 1 μM. The identity of the adrenergic receptors involved in NE-dependent changes in V_m or current was examined pharmacologically. Treatment with the mixed agonist epinephrine (10 sec, 10 μM) and the α-adrenoceptor agonist phenylephrine (10–20 sec, 1 μM) mimicked the effects of NE treatment (Figs. 2F and 3B). In addition, the effect of NE was abolished in cells bathed in medium containing the α_1-adrenoceptor antagonist 1 μM prazosin, (Fig. 3C). However, the β_1-adrenoreceptor agonist ISOP (1 μM) failed to trigger current response (Fig. 3B).

Furthermore, the sustained NE-induced cell depolarization was also abolished by treatment with 1 μM prazosin (Fig. 2E), indicating that this effect is mediated by an α_1-adrenoreceptor. The specific subtype of α_1-adrenoreceptor involved is assumed to be the α_{1B}, because it is highly expressed in the rat pineal gland, and expression of other α-adrenergic receptors in this tissue is undetectable based on microarray analysis (7) and RNA-Seq (Klein, D. C., unpublished data).

The NE-induced current was absent in whole-cell recording with broken membrane (data not shown), which is consistent with the interpretation that the current changes observed in intact cells are mediated by a metabotropic-type receptor.

Role of Ca^{2+}/PLC signaling in NE-induced hyperpolarization

The available evidence indicates that α_{1B}-adrenoreceptors regulate Ca^{2+} levels and PLC activity in the pinealocytes, as discussed in the *Introduction*. This, combined with the findings that cAMP elevating agents, including ISOP (Fig. 3B.) and forskolin (Zemkova, H., unpublished data), do not alter V_m, directed our focus to the

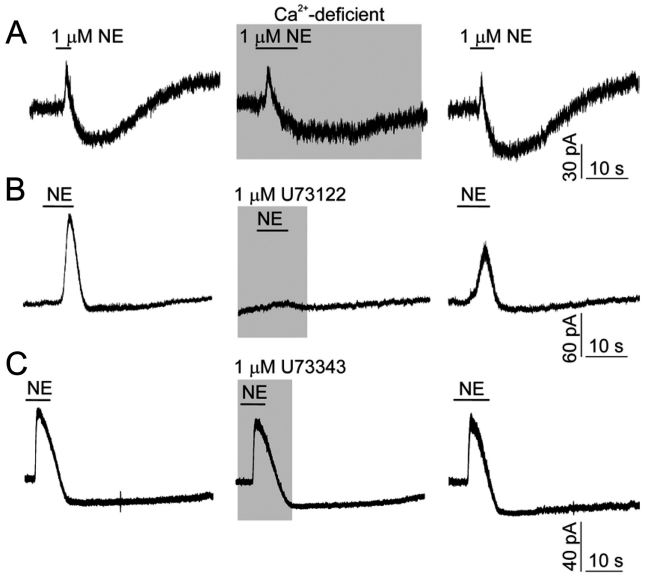

FIG. 4. Dependence of NE-induced biphasic currents on PLC signaling pathway. A, NE-induced biphasic current in pineal cells bathed in calcium-deficient medium. *Shaded area* indicates duration of calcium-deficient medium application. B and C, Inhibition of NE (1 μM)-induced current by 1-min pretreatment of cells with U73122 (1 μM), a PLC inhibitor (B), and the lack of inhibition when cells were treated for the same time with the inactive analog U73343 (1 μM) (C).

roles that Ca^{2+} and PLC play in mediating adrenergic changes in V_m.

The relevance of Ca^{2+} mobilization from endoplasmic reticulum on NE-induced changes in V_m was studied by measuring NE-stimulated currents in the presence and absence of extracellular Ca^{2+}. Removal of extracellular Ca^{2+} had no effect on the amplitude of NE-induced outward current but reduced the amplitude of inward current by 55 ± 17% (Fig. 4A and Table 1). This establishes that Ca^{2+} influx is not essential for the transient hyperpolarization but clearly contributes to the second phase of the NE-induced response, consistent with evidence that NE causes a sustained and Ca^{2+} influx-dependent elevation of $[Ca^{2+}]_i$ (6, 31).

To further test the relevance of Ca^{2+} mobilization from intracellular stores on the NE-induced current response, we used the aminosteroid U73122, which specifically blocks PLC, the activity of which is elevated in the pineal gland by α_1-adrenoreceptor activation (9, 11, 12). Pretreatment of pinealocytes with 1 μM U73122 for 60–120 sec almost completely abolished the NE-induced biphasic current (Fig. 4B). In contrast, the inactive analog U73343 had no effect (Fig. 4C and Table 1). This suggests that PLC is involved in NE-induced hyperpolarization of the plasma membrane by releasing Ca^{2+} stored in endoplasmic reticulum as well as the sustained depolarization by facilitating cation influx, a well described pattern of signaling for Ca^{2+}-mobilizing G protein-coupled receptors (32).

K_{Ca} and nonselective cationic channels contribute to NE-induced biphasic currents

The dependence of NE-evoked outward current on Ca^{2+} mobilization from intracellular stores is consistent with a role of K_{Ca} channels in hyperpolarization of the plasma membrane of pinealocytes. If so, the amplitude of the current should depend on the V_m. To test this, cells were clamped at different potential, and the amplitude of outward current was measured. Figure 5A shows that the amplitude of NE-induced outward current decreased at

TABLE 1. The effect of experimental treatments on the amplitude of the NE-induced current

	Outward current (pA)			Inward current (pA)		
Treatment	NE before	NE+ treatment	NE after	NE before	NE+ treatment	NE after
None (n = 10)	55 ± 16	49 ± 15	46 ± 18	−10 ± 2	−12 ± 2	−11 ± 2
200 nM apamin (n = 8)	56 ± 26	45 ± 18	38 ± 23	−16 ± 3	−17 ± 5	−19 ± 5
30 μM BMI (n = 4)	71 ± 25	29 ± 13, P = 0.028	53 ± 25	−10 ± 4	−11 ± 3	−9 ± 2
30 μM BMI+ 200 nM apamin (n = 3)	30 ± 12	13 ± 3, P = 0.030	26 ± 10	−12 ± 3	−17 ± 5	−19 ± 4
Ca^{2+}-deficient media (n = 6)	33 ± 17	35 ± 13	18 ± 8	−15 ± 4	−6 ± 3, P = 0.011	−16 ± 3
100 nM IBTX (n = 3)	54 ± 24	49 ± 23	27 ± 12	−24 ± 11	−26 ± 9	−26 ± 6
1 μM NIF (n = 3)	19 ± 11	23 ± 10	17 ± 8	−13 ± 5	−14 ± 3	−14 ± 7
1 μM TRAM 34 (n = 4)	47 ± 7	46 ± 12	40 ± 8	−19 ± 4	−20 ± 8	−17 ± 10
1 μM U73343 n = 3)	38 ± 9	44 ± 10	47 ± 9	−13 ± 3	−15 ± 5	−12 ± 6
1 μM U73122 (n = 4)	51 ± 18	8 ± 5, P = 0.029	16 ± 11	−12 ± 4	−2 ± 1, P = 0.003	−6 ± 4
1 μM XE991 (n = 6)	36 ± 8	32 ± 11	31 ± 10	−6 ± 1	−10 ± 4	−9 ± 5

NE (1 μM) was applied three times, once in the presence of drug. Drugs were preapplied for 10–60 sec. Apamin and BMI, specific inhibitors of $K_{Ca}2$ channels; IBTX, specific inhibitor of $K_{Ca}1.1$ channels; TRAM 34, an inhibitor of IK $K_{Ca}1$ and K_{Ca} 3.1 channels; XE991, a KCNQ channel blocker; nifedipine (NIF), antagonist of L-type Ca_v channels; U73343, structural inactive analog of specific phospholipase C inhibitor; U73122, specific phospholipase C inhibitor. Analysis was performed on three to eight cells per treatment. Outward and inward peak current values were obtained approximately 10 and approximately 25 sec, respectively, after NE application. Data shown are mean ± SEM, P values were estimated by a Student's t-test.

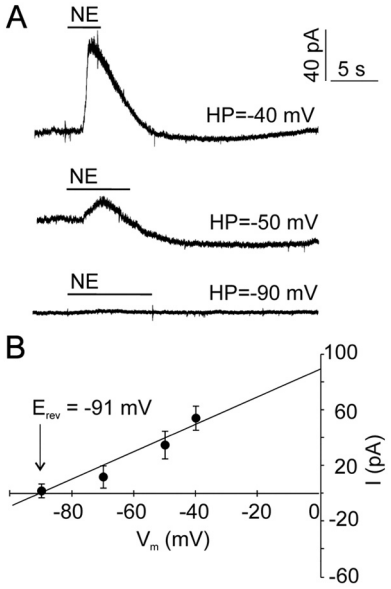

FIG. 5. Voltage dependence of NE-induced outward current. A, Effect of the HP on the amplitude of NE (1 μM)-induced current responses in pineal cells. Notice that the maximum amplitude of NE-induced current decreased at negative HP. B, The reversal potential (E_{rev}) of NE-induced rapid current (I) of -91 ± 5 mV.

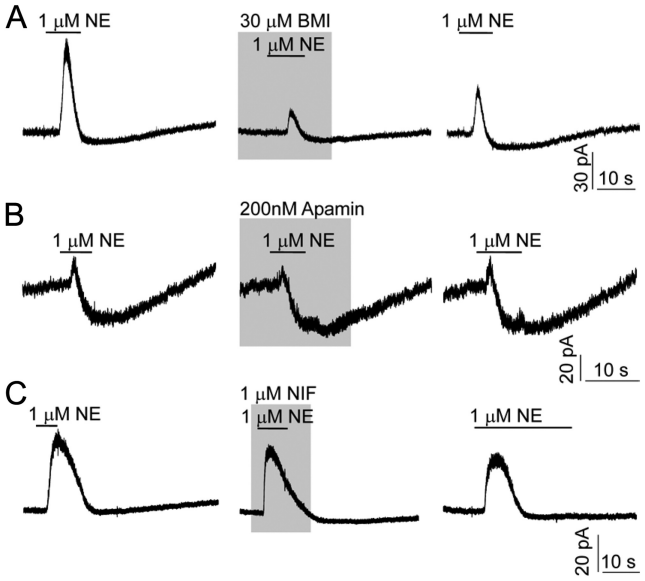

FIG. 6. Characterization of NE-induced outward current. A and B, Inhibition of NE-induced current by K_{Ca} channel blockers. A, Effects of BMI (30 μM), a $K_{Ca}2$ channels blocker, on NE-induced outward current. B, Effects of apamin (200 nM), a SK-K_{Ca} channel blocker, on NE-induced current response. C, Independence of NE-induced outward current of Ca^{2+} influx through nifedipine (NIF)-sensitive L-type Ca_v channels.

HP negative to -40 mV. The estimated reversal potential for this current was -91 ± 5 mV (n = 3), near to the calculated reversal potential for K^+ (Fig. 5B). These results confirm that K_{Ca} channels account for NE-induced hyperpolarization of the cell membrane.

The K_{Ca} channels are composed of two families. One includes three small potassium (SK) channels ($K_{Ca}2.1$, $K_{Ca}2.2$, and $K_{Ca}2.3$) and one intermediate conductance (IK) channel ($K_{Ca}3.1$). The BK-type channels represent the other family of K_{Ca} channels only distantly related to SK and IK channels ($K_{Ca}1.1$, $K_{Ca}4.1$, and $K_{Ca}5.1$) (26). The nature of the channel or channels involved in NE-mediated hyperpolarization was examined using selective K_{Ca} channel blockers under voltage-clamp conditions. At V_m -40 mV, BMI (30 μM), the $K_{Ca}2$ blocker, rapidly and reversibly blocked NE-evoked outward current by $59 \pm 18\%$ (Table 1 and Fig. 6A). The apamin-sensitive component of inward current was also identified (Fig. 6B and Table 1). The NE-induced current was insensitive to IBTX, a specific inhibitor for $K_{Ca}1.1$ channels, TRAM 34, a blocker of $K_{Ca}1$ and $K_{Ca}3.1$, and the potassium voltage-gated and cyclic nucleotide-regulated channel, subfamily Q, member 7 (Kv7/KCNQ) inhibitor XE991 (Table 1).

Nifedipine (1 μM), an L-type Ca_v channel antagonist, had no effect on NE-stimulated outward and inward currents (Fig. 6C and Table 1), suggesting that Ca^{2+} influx through these channels is not critical for a transient hyperpolarization and sustained plasma membrane depolarization. This is in contrast to the role these channels play in spontaneous firing of AP and the accompanied Ca^{2+} influx. Because the sustained depolarization was abolished in cells bathed in Na^+-deficient medium (data not shown) and partially inhibited by removal of bath Ca^{2+} (Table 1), these results indicate that the inward current is mediated by one or more nonselective cationic channels.

Discussion

In this study, the electrophysiological properties of single pinealocytes have been examined using amphotericin B-perforated patch clamp recording. The enhanced sensitivity and responsivity to NE of the technique has provided us with new insights into the electrical characteristics of these cells as discussed below.

Spontaneous electrical activity in resting cells

Consistent with published *in vivo* and *in vitro* studies (2, 24, 25, 27), we observed that pineal cells spontaneously fire AP. Firing of AP requires the expression of several subtypes of depolarizing and hyperpolarizing channels. Chicken pinealocytes express the depolarizing Na_v channels (17), but it is unlikely that they play a role in spontaneous electrical activity. First, the resting V_m in these cells is more depolarized than in other cell neuroendocrine types, approximately -36 to -46 mV in recordings by several groups (28, 33, 34), values at which the majority of these channels are inactivated. Second, tetrodotoxin, a blocker of Na_v channels, does not abolish spon-

taneous firing of AP in rat pinealocytes (27), suggesting a role of Ca_v channels in spike depolarization. Consistent with this hypothesis, it has been reported that rat and chicken pineal cells express L-, P/Q, and N-type Ca_v channels (17, 18, 35, 36). Unpublished RNA transcriptome profiling by RNA-Seq indicates that relative expression of mRNA encoding these channels in rat pinealocytes is L-type > T-type ≫ P/Q-type and ≈ R-type channels (Klein, D. C., unpublished data). The L-type Ca_v channels in pinealocytes are also subject of regulation by phosphorylation (20, 35, 37). Pinealocytes also express several K^+ channels, activation of which is needed to restore the resting V_m (19, 38). We found that rapid depolarization (removal of Na^+-free solution) was able to increase the frequency of AP, but no effect was observed after NE application. This may be explained by the participation of ion channels, which are involved in generation of AP that are not directly regulated by α_{1B}-drenoreceptor signaling. Alternatively, a low resting V_m and a slow onset of depolarization evoked by NE (see below) may have inactivated voltage-gated channels before firing of AP could start. Further experiments are needed to distinguish between these two possibilities and to explore the role of NE in electrical excitability of pinealocytes.

The low resting V_m is of potential physiological relevance, because it permits steady-state Ca^{2+} influx through nondesensitizing Ca_v channels in nonfiring cells. Our results also indicate that high resting Na^+ conductance accounts at least partially for elevated resting V_m of pineal cells, as is also seen in secretory pituitary cells, where removal of bath Na^+ has a hyperpolarizing effect (39). At the present time, it is unknown whether ion channel(s) or transport mechanism(s) are responsible for such constitutive active Na^+ conductance, referred to as "the background Na^+ conductance" (40).

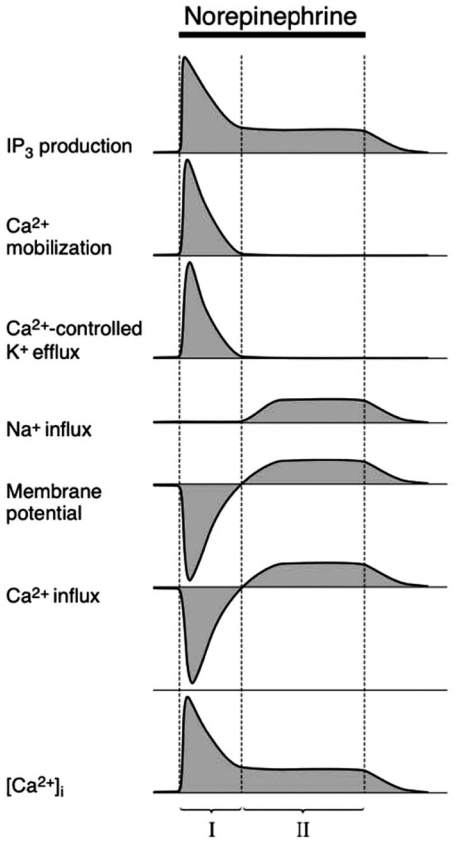

FIG. 7. Schematic representation of α_{1B}-adrenoceptor actions in pinealocytes. Phase I, *top to bottom*, Addition of NE (*horizontal bar*) induced a rapid and sustained PLC activation, leading to generation of inositol trisphophate (IP_3) (*top panel*) and diacylglycerol (data not shown). The released IP_3 activates its receptors, causing Ca^{2+} mobilization from endoplasmic reticulum (*second panel from top*), activation of K_{Ca} channels (*third panel*), K_{Ca}-dependent hyperpolarization of cell membrane (*fifth panel*), and inhibition of Ca^{2+} influx through Ca_v channels (*second panel from bottom panel*). Phase II, *top to bottom*, The sustained PLC signaling leads to activation of nonselective cationic channels (*fourth panel*), causing sustained cell membrane depolarization (*fifth panel*). The permeability of these channels to Ca^{2+} accounts for the sustained rise in $[Ca^{2+}]_i$ (*second panel from bottom*), and the transient Ca^{2+} mobilization accompanied with sustained Ca^{2+} influx accounts for the biphasic Ca^{2+} signals (*bottom panel*).

α_{1B}-Adrenergic receptor-induced biphasic change in V_m

Activation of the pineal α_{1B}-adrenoreceptor facilitates a transient Ca^{2+} release from intracellular pools and a spike in $[Ca^{2+}]_i$. After this, there is a sustained elevation of $[Ca^{2+}]_i$ dependent upon Ca^{2+} influx (6, 10, 31, 33, 41). Furthermore, the NE-induced Ca^{2+} influx was not found to be blocked by 1 μM nifedipine, arguing against the role of L-type Ca_v channels. In our hands, a short application of NE (10–20 sec, 1 or 10 μM) elicited a transient and robust outward current, which in current clamp recording could be seen as a transient hyperpolarization, followed by a smaller inward current, which could be seen as a sustained depolarization (Fig. 7). We also found that both NE-induced currents were inhibited by prazosin and mimicked by phenylephrine and epinephrine, but not by ISOP, which indicates that the NE-induced biphasic response is mediated by α_{1B}-adrenoreceptors. The observation that similar responses were observed with PE, epinephrine, and NE, but not by ISOP, suggests that the NE-induced changes in V_m described here are purely α_{1B}-adrenergic.

The NE ($ED_{50} \sim 1$ μM)-induced outward current was also seen in cells bathed in Ca^{2+}-deficient medium, indicating that Ca^{2+} mobilization from an intracellular pool generates the hyperpolarizing current. This current had reversal potentials close to the K^+ equilibrium potential, indicating that the NE-induced Ca^{2+} release from intracellular pool activates K_{Ca} channels, consistent with previous reports (21, 33). Our pharmacological studies suggest SK-type channels sensitive to BMI and apamin contribute 60% to Ca^{2+}-dependent hyperpolarization. In

contrast, our pharmacological studies with TRAM 34, a highly selective blocker of IK Ca^{2+}-activated K^+ channels, and XE991, a selective inhibitor of Kv7/KCNQ channels (42), fail to provide evidence that these channels contribute to the NE-induced outward current (Table 1).

The finding that the NE-induced outward current was blocked by a selective inhibitor of PLC indicates that activation of this enzyme is required for Ca^{2+} mobilization, as has been widely observed in other systems. It is of interest to note that pineal PLC is sensitive to Ca^{2+} within the physiological range of this cation (11). As a result, it seems likely that the initial receptor-dependent activation of PLC results in immediate positive feedback via Ca^{2+}, which contributes to sustained activation of this enzyme.

Previous single channel recordings revealed the presence of the BK-type K_{Ca} channels in pineal cells activated by NE (21), which are blockable by scorpion toxin (21) and tetraethylammonium (17). It was also claimed that these channels are activated by NE-induced Ca^{2+} influx (33). Consistent with this, we found that the transient NE-induced K_{Ca} current was insensitive to IBTX, indicating that Ca^{2+} released from intracellular stores cannot activate BK channels. Similarly, pituitary cells express both BK and SK channels, and Ca^{2+} mobilization activates SK channels, whereas BK channels are activated by Ca^{2+} influx (26).

As observed with the NE-induced hyperpolarization, the sustained depolarizing current did not develop when PLC was inhibited, supporting the interpretation that PLC activity regulates both Ca^{2+} mobilization and the sustained Ca^{2+} influx current. The inward current was significantly attenuated by removal of bath Ca^{2+}, suggesting that Ca^{2+} mobilization is linked to the sustained Ca^{2+} influx. The nature of this link is not clear. In general, in nonexcitable cells, Ca^{2+} mobilization is coupled to Ca^{2+} influx through Orai channels by STIM proteins in endoplasmic reticulum, a process referred to as "capacitative calcium entry" through store-operated Ca^{2+} channels (43, 44). In pinealocytes, store-operated Ca^{2+} channels have been suggested to account for the NE-induced rise in $[Ca^{2+}]_i$ (33). However, it is unlikely that these channels account for sustained depolarization, because Orai channels are highly selective for Ca^{2+}, whereas in our hands, the sustained depolarization and inward current were only partially abolished by removal of bath Ca^{2+}. This suggests that a depolarizing channel is probably permeable to both Na^+ and Ca^{2+}. This is similar to the role that NE-activated depolarizing nonselective cation channels play in controlling Ca^{2+} influx (16). Furthermore, it is now well established that TRPC, transient receptor potential classic channel, can be activated via Ca^{2+}-mobilizing receptors via a PLC mechanism and that these channels conduct both Na^+ and Ca^{2+} (45). It is of interest to note that the gene encoding the scafolding Trpc4, transient receptor potential classic channel member 4, associated protein is highly expressed in the pineal gland, relative to other channel proteins (Klein, D. C., unpublished data); this protein might play a critical role in controlling the inward current.

As indicated above, previous studies have reported an adrenergically generated hyperpolarization in the rat pineal gland. However, none have indicated that this is transient in nature nor that it is followed by depolarization. The failure of previous investigations to observe depolarization may reflect technical differences in recording methods, which precluded detection. In our study, the biphasic response was observed in 67% of voltage-clamped cells. The failure to observe this pattern consistently is likely to reflect variable experimental conditions beyond our control, cell preparation-induced decrease in the receptor or channel levels, or natural variations in the expression of receptors or channels. Consistent with this is the finding that there is significant cell-to-cell variation in the amplitude of the biphasic calcium response of rat pinealocytes to NE (15). In addition, observations made on single isolated pinealocytes used in the current studies may differ from those made using the intact gland because cell-cell communication through gap junctions (46) might influence observed electrophysiological changes. Cell-cell communication, combined with the influence of extracellular space retention of ions within the cultured intact gland, could buffer electrophysiological responses of single cells and delay depolarization (28, 29). Depolarization was also observed in studies performed in cultured pineal organs of guinea pigs (47).

V_m and pinealocyte biology

Our findings make it clear that adrenergic activation of the pinealocyte produces prolonged depolarization, leading to the conclusion that this is necessary for ideal adrenergic activation of the pinealocyte. However, the evidence of a prolonged depolarization puts in question the view that sustained hyperpolarization, not depolarization, is required for the hours long stimulation of AANAT (28, 48). This view was based on the finding that experimental depolarizing treatments (ouabain, $ED_{50} \sim 0.25$ μM; K^+, $ED_{50} \sim 35$ mM) block NE and cAMP stimulation of this response (28, 48). One explanation for these effects is that these experimental depolarization treatments may overwhelm the capacity of the pinealocyte to maintain a level of physiology required to generate the AANAT response.

A related issue is the physiological relevance of the sustained depolarization, in addition to the associated elevation of $[Ca^{2+}]_i$, which would appear to reflect signaling.

One can consider that the V_m maintained by adrenergic activation of the pinealocyte is broadly related to cell physiology through solute transport, which is mediated by panoply of solute transporters. The dependence of transporter function on V_m is consistent with the view that the sustained depolarization produced by NE establishes ideal electrochemical conditions allowing pinealocyte transporters to operate in an optimally integrated manner in support of cellular metabolism.

Another issue of interest is the functional significance of the initial transient hyperpolarization. One possible explanation is that this blocks Ca^{2+} influx through voltage-gated channels, thereby making it easier for Ca^{2+} pumps to control $[Ca^{2+}]_i$.

Summary

The results of the studies presented here indicate that there is a Ca^{2+} signaling pathway operative in rat pinealocytes controlled by α_{1B}-adrenoreceptors, the activation of which causes two sequential changes in several plasma membrane and intracellular events (labeled in Fig. 7 as I and II). These receptors stimulate PLC, leading to an increase in inositol trisphosphate production and Ca^{2+} mobilization, as well as an increase in diacylglycerol production and activation of protein kinase C. Calcium mobilization in turn activates a mixture of K_{Ca} channels expressed in these cells. A substantial part of this current passes through SK channels that have been identified here for the first time as novel effectors for NE receptors in pinealocytes. Ca^{2+} influx through Ca_v channels is silenced due to cell membrane hyperpolarization. Mobilization rapidly depletes intracellular Ca^{2+} stores, thereby terminating the activity of K_{Ca} channels. During sustained NE application, there is a PLC-dependent activation of nonselective cation channels, permitting Ca^{2+} influx, resulting in membrane depolarization. Enhanced Ca^{2+} influx accounts for a sustained elevation of $[Ca^{2+}]_i$, which serves to enhance adrenergic activation of PLC and to facilitate the increase in arylalkylamine N-acetyltransferase activity and melatonin production.

A feature of this scenario is that Ca^{2+} from intracellular stores regulates SK type of K_{Ca} channels, whereas Ca^{2+} from influx does not, as has been established for these channels in other systems (49), apparently reflecting differences in localization/compartmentalization of SK and BK channels and in sensitivity to Ca^{2+}. The sustained PLC activity, as indicated by the plateau in inositol trisphosphate production in Fig. 7, *top panel* (II), is thought to activate several canonical transient receptor potential channels as seen in other systems. Diacylglycerol may play a role in this process (45). Future studies should clarify whether this is the case with NE-stimulated pinealocytes, thereby expanding our knowledge of the central role that PLC and Ca^{2+} play in mediating adrenergic activation of these cells.

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